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Effect of Recombinant Human Erythropoietin Treatment on Circulating Reticulated Platelets in Uremic Patients: Association With Early Improvement in Platelet Function

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Recombinant human erythropoietin improves platelet function in uremia through the correction of anemia, but this effect can be seen also before the hematocrit rise. We studied 12 hemodialyzed patients (seven men, five women) who received recombinant human erythropoietin (40 IU kg^{-1} i.v., three times weekly) and were evaluated before treatment and after three doses; 24 control subjects were used. Platelet aggregation induced by adenosine 5′-diphosphate (ADP), epinephrine, collagen, arachidonic acid, and ristocetin, and reticulated platelets determined by flow cytometry after staining with thiazole orange were measured. Platelet aggregation induced by all the agonists were impaired in uremic patients ($P < 0.01$), but ADP and ristocetin-induced aggregations improved after treatment ($P < 0.01$). Hemodialyzed patients had less reticulated platelets than controls ($P < 0.01$). Reticulated platelets increased after three doses of treatment ($P < 0.01$). In conclusion, improvement of platelet function at early stages of recombinant human erythropoietin treatment may be attributed to the increase in young platelets detected as reticulated platelets. Am. J. Hematol. 59:105–109, 1998.

Key words: platelet function; recombinant human erythropoietin; uremia; reticulated platelets; flow cytometry; thiazole orange

INTRODUCTION

Patients with chronic renal failure have a bleeding tendency that has been ascribed both to plasmatic and platelet defects, resulting in impaired interaction of platelets with vessel subendothelium and defective aggregate formation [1–3]. The anemia frequently found in uremic patients has been associated also with the hemostatic defect in these patients [4,5]. Recombinant human erythropoietin (rHuEPO) effectively corrects the anemia of uremic patients and improves platelet function ‘‘in vivo,’’ mainly through increasing hematocrit and blood viscosity [6,7]. Furthermore, improvement of platelet aggregability has been reported during rHuEPO treatment [7–10] and amelioration of platelet function has also been observed in the early stages of treatment, when an effect on hematocrit is still not reached [10,11]. The mechanisms of such early improvement of platelet function with rHuEPO are unknown. In a previous report, our group suggested a release to the blood of young platelets, which are metabolically more active, to explain this fact [10]. Young platelets can be identified by their increased ribonucleic acid (RNA) contents [12]. Staining platelets with thiazole orange, a dye that can enter living cells and binds to RNAs, allows the identification of RNA-rich (reticulated) platelets by flow cytometry [12–16]. In the present study we used thiazole orange staining and flow cytometry techniques to evaluate the effect of rHuEPO treatment for correction of renal anemia on reticulated platelets and on platelet function in the early stages of treatment.

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PATIENTS AND METHODS

Patients

Twelve patients with end-stage renal disease (seven men and five women) were included in the study. Mean age (±SD) was 42.7 ± 18.9 years (range 19–68 years) and mean time on hemodialysis treatment was 59.8 ± 39.0 months. Causes of renal failure included chronic glomerulonephritis (four), nephroangiosclerosis (two), polycystic kidney disease (two), chronic pyelonephritis (two), diabetic nephropathy (one), and unknown (one). All of them were undergoing hemodialysis three times weekly. They were treated for four hr through a wrist or a brachial arteriovenous fistula by using 1.1–1.5 m^2 hollow fiber cellulose acetate dialysers (CA 110–CA 150, Nissho Corp., Osaka, Japan) and a bicarbonate-containing buffer dialysate (Nefro-ion, Mollerusa, Spain). Additionally, 24 healthy control subjects (16 men and 10 women, mean age ± SD = 40.3 ± 11.7 years) were studied. Coagulation tests (prothrombin time, partial thromboplastin time, and fibrinogen levels) were within the normal range in all patients and control subjects. None of them were taking medication known to affect platelet function and none had received blood-related products during the period of study or in the previous two weeks.

Patients received rHuEPO (Erantin®, Boehringer Mannheim, Mannheim, Germany) at an initial dose of 40 IU kg^{-1} i.v. three times weekly at the end of the dialysis session and were evaluated before starting the rHuEPO and after receiving three doses. No thrombotic episodes were seen in the patients.

Informed consent was obtained from all the participants, and the study, approved by the Human Experimental Committee, was performed according to the principles of the declaration of Helsinki.

Samples

Blood samples were obtained by clean venipuncture without veno-occlusion. Samples were obtained just before the first dose of rHuEPO and seven days after the beginning of the treatment. In all the cases, samples were obtained immediately before the second hemodialysis procedure of the week and before receiving heparin. Determinations included complete blood cell counts, platelet aggregation ''in vitro'' induced by several agonists, and reticulated platelets determined by flow cytometry.

Samples for flow cytometry and blood cell counts were drawn in four mmol L^{-1} tri-potassium ethylenediaminetetraacetic acid (EDTA) tubes (Becton Dickinson, Rutherford, NJ). Blood samples for aggregation studies were collected by using citrate-phosphate-dextrose (CPD) (19 mmol L^{-1} final citrate concentration) as anticoagulant.

Blood Cells Counts and Platelet Aggregation Studies

Blood cell counts were performed in an automatic electronic counter (Technicon H-1 System, Technicon Instruments, Tarrytown, NY).

Platelet aggregation studies were done in freshly prepared platelet-rich plasma obtained by centrifugation (10 min, 250g, 22°C) in a Lumi-aggregometer (Chrono-Log, Havertown, PA). Platelet-rich plasma was adjusted to 240–260 × 10^9 platelets L^{-1} and the following agonists were used: ADP, two μM (Kyoto Dai-Ichi Kagaku, Kyoto, Japan); epinephrine, 10 μM (Menarini Diagnostica, Milano, Italy); collagen, five μg mL^{-1} (Menarini Diagnostica, Milano, Italy); arachidonic acid, 1.4 mM (Menarini Diagnostica, Milano, Italy); and ristocetin, one mg mL^{-1} (Lundbeck, Copenhagen, Denmark).

Flow Cytometry

Platelets were stained for RNA content by using the dye thiazole orange as described previously [16]. Briefly, whole blood samples were fixed with 1% paraformaldehyde, washed with phosphate buffered saline pH 7.2 (Bio-Mérieux, Marcy-l'Etoile, France) plus 1% bovine serum albumin (Sigma, St. Louis, MO), and 1% heat inactivated AB-group human serum, and incubated with a monoclonal antibody anti-glycoprotein Ib (CD42b) (Janssen Biochimica, Geel, Belgium) (30 min, 22°C), washed and incubated with a phycoerythrin-tagged goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) (30 min, 22°C). After a new washing, platelets were incubated with one mL of thiazole orange (Becton Dickinson, San Jose, CA) (one hr, 22°C) and samples were read in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) by using Lysys II software (Becton Dickinson). Platelets were selected in logarithmic amplification by forward scatter, side scatter, and 585 nm fluorescence (phycoerythrin) gates and read at 530 nm for thiazole orange fluorescence. An unstained control sample was used to determine autofluorescence and instrument background. Thiazole-orange–stained platelets with fluorescence higher than the 99% of the unstained control samples were considered to be reticulated platelets. In each sample, 10,000 platelets were analyzed, the percentage of reticulated platelets recorded, and the absolute number of reticulated platelets was calculated by using the percentage and the platelet count. The intra-assay coefficient of variation was 7.5%.

Statistical Analysis

Results are expressed as mean ± SD. Either the Mann Whitney test or Wilcoxon's test for paired data was used for comparisons between groups. The relationship between the level of reticulated platelets and platelet function was evaluated analyzing the correlation between the

increase after rHuEPO treatment in the percentages of reticulated platelets and the increase in the ADP-induced aggregations by the Spearman rank-order correlation test. A P level of 0.05 was considered as statistically significant.

RESULTS

Blood Cells Counts

There were no significant differences in platelet counts between patients before treatment and control subjects ($203.6 \pm 82.9 \times 10^9\ L^{-1}$ vs. $237.8 \pm 68.4 \times 10^9\ L^{-1}$, $P = 0.2$). Platelet counts showed no significant increase in the samples obtained after seven days on rHuEPO treatment ($207.6 \pm 70.1 \times 10^9\ L^{-1}$, $P = 0.9$).

At the start of rHuEPO therapy, mean patients' hematocrit was $0.22 \pm 0.03\ L\ L^{-1}$. Hematocrit values were no different in the samples obtained after three doses of rHuEPO ($0.22 \pm 0.04\ L\ L^{-1}$, $P = 1.0$).

Aggregation Studies

Aggregation studies in uremic patients showed a significantly decreased response to all the agonists (ADP, 40.2% ± 13.4%; ristocetin, 48.6% ± 16.5%; arachidonic acid, 55.7% ± 15.6%, collagen, 60.7% ± 16.7%; and epinephrine, 52.2% ± 18.3%) when compared with control subjects (ADP, 84.1% ± 16.7%; ristocetin, 85.8% ± 16.5%; arachidonic acid, 86.1% ± 12.7%; collagen, 88.6% ± 11.9%; and epinephrine, 79.5% ± 17.4%, $P < 0.01$). Aggregation studies performed after one week on rHuEPO treatment showed a significant increase in ADP and ristocetin-induced aggregations (ADP, 56.7% ± 15.3%; ristocetin, 60.1% ± 17.8%, both $P < 0.01$) compared with pretreatment samples. No differences were seen between pre- and post-rHuEPO samples when arachidonic acid (59.7% ± 13.6%), collagen (61.2% ± 14.4%) or epinephrine (52.8% ± 19.1%) were used as agonists.

Reticulated Platelets

In the samples obtained previous to rHuEPO treatment, the percentage and absolute number of reticulated platelets were significantly lower in hemodialyzed patients (3.2% ± 1.3%, and $6.6 \pm 2.6 \times 10^9\ L^{-1}$) than in control subjects (8.9% ± 3.4%, and $21.2 \pm 6.2 \times 10^9\ L^{-1}$, both $P < 0.01$) (Figures 1 and 2). Reticulated platelets significantly increased in the samples obtained after one week under rHuEPO treatment considering both percentage (6.3% ± 2.3%, $P < 0.01$) and absolute counts ($13.1 \pm 4.6 \times 10^9\ L^{-1}$, $P < 0.01$) (Figures 1 and 2).

A significant correlation was observed between the increases after rHuEPO treatment in the percentages of reticulated platelets and in the ADP-induced platelet aggregations ($P < 0.01$).

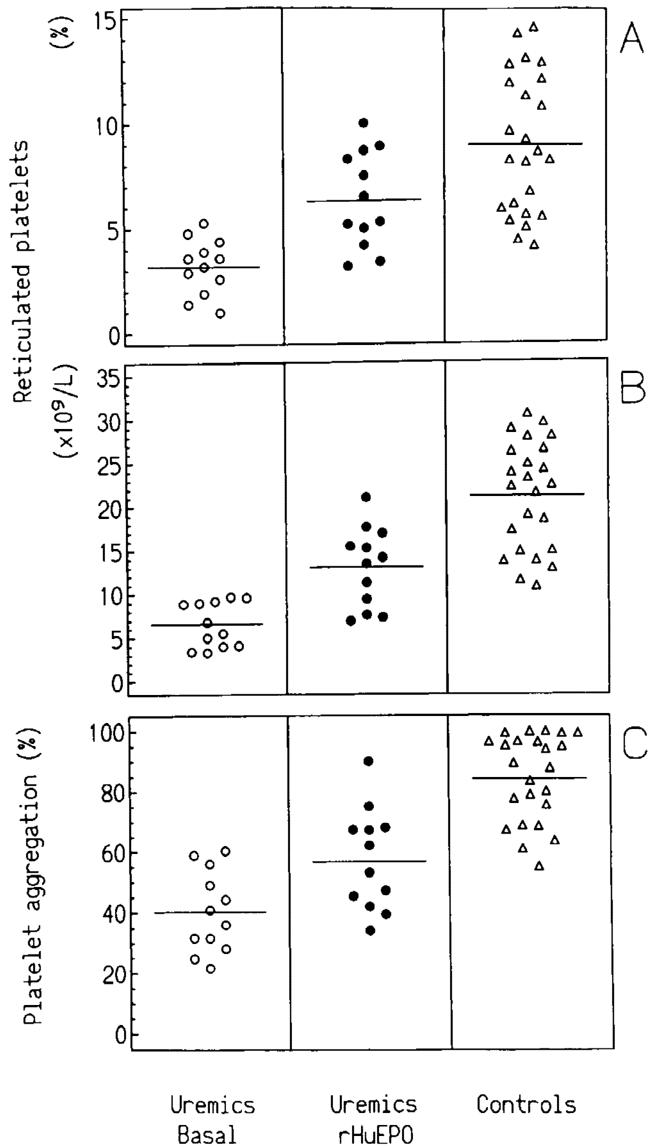

Fig. 1. The graph shows the percentages of reticulated platelets (A), the absolute numbers of reticulated platelets (B), and the ADP-induced platelet aggregations (C) seen in 12 anemic patients with end-stage renal disease before receiving rHuEPO (uremics basal, open circles), in the same patients after seven days of treatment (uremics rHuEPO, solid circles), and in 24 normal individuals (controls, open triangles). Horizontal lines are means. Reticulated platelets and ADP-induced aggregations were significantly lower in uremic patients ($P < 0.01$) than in control subjects, and increased after rHuEPO treatment ($P < 0.01$).

DISCUSSION

In the present study, the early effects of rHuEPO treatment for correction of renal anemia on platelets was evaluated by measuring the platelet RNA content and the platelet aggregation. According to our results, rHuEPO treatment increased the number of reticulated platelets in uremic patients and improved platelet function in the first

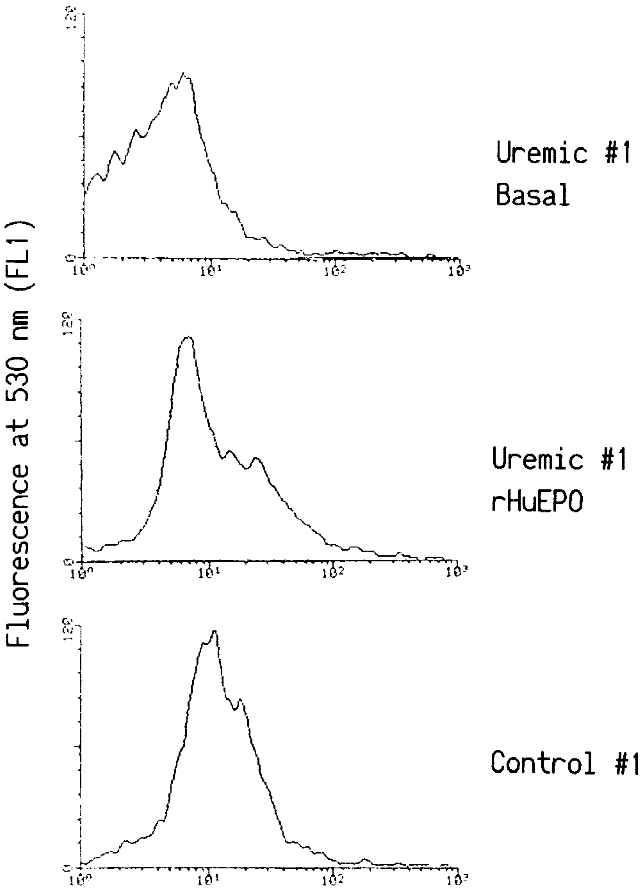

Fig. 2. Flow cytometry graphs of fluorescence at 530 nm (thiazole orange fluorescence) seen in a uremic patient before receiving rHuEPO (uremic #1 basal), in the same patient after seven days of treatment (uremic #1 rHuEPO), and in a control individual (control #1). Thiazole-orange–stained platelets with fluorescence higher than the 99% of an unstained sample used as negative control were considered to be reticulated platelets.

week of treatment, before any correction of the anemia was noticeable. Chronic renal failure is often associated with bleeding disorders, indicated by decreased platelet aggregation and prolonged bleeding time. The hemostatic defect in uremia has been related to several platelet and plasmatic factors [1–3]. In addition, anemia associated with severe chronic renal failure is another factor influencing the uremic platelet defect because red cells can enhance platelet function by both biochemical and rheological mechanisms [4,5,17]. rHuEPO treatment corrects anemia in uremic patients and improves primary hemostasis through the hematocrit rise [6,7]. Although anemia is an important factor, it is not the sole determinant of uremic bleeding because rHuEPO can improve platelet function independently of the effect of rHuEPO on hematocrit [8–11]. We confirmed an improvement in platelet response to several agonists detected after only three doses of rHuEPO and before any effect on hematocrit was observed. Moreover, we detected at the same time changes in the platelet subpopulation patterns as determined by platelet RNA content by using flow cytometry. After seven days under treatment with rHuEPO, we detected a significant increase in the proportion of reticulated platelets that may reflect a modification of the platelet turnover. Reticulated platelets are the youngest platelets in circulation as demonstrated by ''in vivo'' biotinylation [18]. Furthermore, young platelets are hemostatically more active than aged platelets [19]. The decreased number of reticulated platelets in pre-rHuEPO samples in hemodialyzed patients in comparison with control subjects may be attributed to an effect of hemodialysis resulting in fragmentation and disappearance of the more reactive platelets [16]. Results obtained in continuous ambulatory peritoneal dialysis and in prehemodialysis chronic renal failure patients suggest that the decreased reticulated platelet percentages can be ascribed mainly to the hemodialysis procedure [16]. The observed improvement in platelet function after starting rHuEPO, seen together with the increase in reticulated platelets, is probably due to an increased release of younger platelets to the circulating blood. In this sense, rHuEPO can act as a humoral growth factor [20] stimulating certain aspects of megakaryocyte colony formation [21,22]. The increases in platelet counts [17] and platelet volume [23] previously observed during rHuEPO treatment also suggest an enhancement of formation and release of young platelets. We did not observe an increase in platelet counts, but this fact can be attributed to the study design evaluating early phases of therapy to avoid the confounding effect of the hematocrit rise. In the same sense, in bone marrow recovery after autologous bone marrow transplantation, the rise in the proportion of reticulated platelets precedes the increase of platelet counts [24].

Some additional known side effects of rHuEPO treatment suggest circulation of more reactive platelets. First, hemodialyzed patients receiving rHuEPO show an increased risk of thrombosis [25–27], particularly of the arteriovenous fistula, even during the initial phases of treatment. Second, an increase in heparin requirement during hemodialysis is frequently detected after the start of rHuEPO therapy [28]. Thus, increased platelet reactivity may be attributed, at least in part, to the rHuEPO-induced rise in younger and biologically more active platelets. In conclusion, rHuEPO treatment for correction of renal anemia improves platelet function at the very early stages of treatment, before the hematocrit rise. This fact may be ascribed to the increase in young platelets detected as reticulated platelets by flow cytometry.

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